

Cold Hardening of *Meloidogyne hapla* Second-stage Juveniles¹

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Abstract: The effect of previous exposure to low temperatures on freezing tolerance was determined for second-stage juveniles of *Meloidogyne hapla*. Juveniles in 5% polyethylene glycol 20,000 were exposed to 0–24 C for 12–96 hours, and then freezing tolerance was assessed by freezing samples at –4 C for 24 hours, thawing, and determining survival. Freezing tolerance was inversely related to prefreeze temperatures of 4–24 C. Prefreeze exposure to 4 C resulted in fourfold greater freezing tolerance than did exposure to 24 C. Mortality occurred during prefreeze exposure to 0 C. Most of the increase in freezing tolerance at 4 C occurred during the first 12 hours. In soil, prefreeze exposure to 4 C resulted in greater freezing tolerance than did prefreeze exposure to 24 C.

Key words: acclimation, cold hardening, cold tolerance, freezing tolerance, *Meloidogyne hapla*.

Poikilotherms inhabiting temperate climates undergo significant physiological changes to survive winter. These changes are referred to as resistance acclimatization or cold hardening and are induced by environmental cues indicative of winter, such as low temperature or short photoperiod (5,6,12,13).

Several studies (2,11,14,16,17) have compared cold tolerance of nematode species or life stages, with little emphasis on hardening. Differences in tolerance between species may not be evident unless they have been hardened properly. Populations may also differ in the elicitors, temporal characteristics, and mechanisms of the cold hardening response.

Objectives of our research were to determine 1) whether previous exposure to low temperatures would increase the freezing tolerance of *Meloidogyne hapla* Chitwood juveniles, 2) the time course of changes in freezing tolerance, and 3) whether previous exposure to low temperatures would increase freezing tolerance in soil. We defined freezing tolerance as the ability to survive a frozen environment.

MATERIALS AND METHODS

Sample preparation: *Meloidogyne hapla*, isolated from ginseng (*Panax quinquefolium* L.) in Wisconsin in 1985, was maintained on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a greenhouse. Nematodes were reared by transplanting 2-week-old tomato seedlings to 15-cm pots containing 10⁴ freshly hatched juveniles in pasteurized Plainfield loamy sand (92% sand, 5% silt, 3% clay). The plants were grown for 5 months in a growth chamber at 24 ± 1 C with a 12-hour photoperiod.

Juveniles were obtained from roots aerated in tap water at room temperature (24 ± 3 C). The water was changed at 12 hours, and juveniles emerging from roots in the subsequent 48 hours were collected on #2 filter paper by filtration with mild suction. The filter was placed on four layers of tissue on a Baermann funnel. After 12 hours, juveniles were collected and rinsed with tap water over a 5-µm nitrocellulose filter (Millipore, Bedford, MA). The juveniles were suspended in 5% (wt:wt) polyethylene glycol 20,000 (Sigma, St. Louis, MO) to a density of 125 individuals/ml, and samples were prepared by delivering 1.0 ml of the suspension to 20-ml test tubes. Samples prepared with nematodes harvested from one plant constituted one experimental block.

General protocol: Samples were placed in incubators or refrigerated ethylene glycol :

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water baths (Neslab, Newington, NH) at specified temperatures and then moved to a bath at -4 C for freezing. A 100- μ l capillary tube containing ice was placed in each sample to initiate freezing. Samples were frozen for 24 hours and then transferred to 4 C for 12–18 hours. After warming to room temperature, samples were individually rinsed into a counting dish and observed at $40\times$ magnification. The percentage of juveniles that survived was determined for each sample on the basis of spontaneous movement or movement induced by tapping with a fiberglass pick near the nerve ring.

Temperatures were measured and recorded hourly with thermocouples and a CR21X datalogger (Campbell Scientific, Logan, UT). Incubator temperatures (mean \pm range) were 0.1 ± 0.1 , 3.1 ± 0.6 , 7.9 ± 0.4 , 12.3 ± 1.4 , 15.8 ± 1.0 , 19.5 ± 0.03 , and 23.8 ± 0.7 C. Sample temperatures in baths were the same as specified, ± 0.1 C.

Effects of prefreeze temperature: Two experiments were conducted. Each block of the first experiment consisted of 49 samples, seven of which were placed in each of the seven incubators at the temperatures listed. At 24, 48, and 96 hours, two samples from each incubator were frozen and thawed. Samples were allowed to warm to room temperature for 2 hours before survival was determined. The one sample left in each incubator served as a nonfrozen control. Five complete blocks were conducted.

Each block of the second experiment had 60 samples, 12 of which were placed in baths at 0, 2, 4, and 8 C, and in an incubator at 24 C. At 12 and 24 hours, five samples were removed from each bath or incubator, and freezing tolerance was measured. The two samples remaining at each temperature served as nonfrozen controls. Five complete blocks were conducted.

Time course of increased freezing tolerance: For each of five complete blocks, 30 samples were placed in a bath at 4 C, and another 30 were placed in an incubator at 24 C. At the same time, an additional five

samples were frozen. At 3, 6, 9, 12, and 24 hours, five samples were removed from each temperature, frozen, and thawed. Samples were warmed to 20 C for 24 hours before survival was assessed. The five samples remaining at 4 and 24 C served as nonfrozen controls.

Increased freezing tolerance in soil: Samples were prepared by placing 5 g Plainfield sand, oven dried at 60 C for 48 hours, in 50-ml glass culture tubes. Approximately 500 juveniles in 2 ml deionized water were added to each tube, followed by an additional 3 g soil. Eighteen samples were prepared for each block, nine of which were put in a bath at 4 C. The remaining nine samples were left at room temperature. After 12 hours, six samples from each temperature were frozen (capillary tubes were not needed for nucleation). The remaining three samples from each temperature served as nonfrozen controls. A modified sucrose centrifugation procedure (7) was used to extract juveniles from the soil. Within 6 hours of extraction, the total number of live and dead juveniles was determined for each sample. Five complete blocks were conducted.

Data analyses: Data analyses for the first three experiments were similar. The two samples in the first experiment (five in the second and third experiments) for each temperature \times time combination within each block were treated as subsamples. Subsample means were analyzed using a blocked split level analysis of variance (ANOVA), with temperature as the main factor and time as the subfactor. The method of least significant differences (LSD) was used for mean comparisons. Because the variance associated with main factor (temperature) effects is different from the variance associated with subfactor (time) effects, separate LSD were calculated for comparing temperature \times time combinations across temperatures or times within any given temperature (10).

The survival of nonfrozen controls in the first two experiments was analyzed by a one-factor ANOVA with blocking. For the second experiment, the two samples

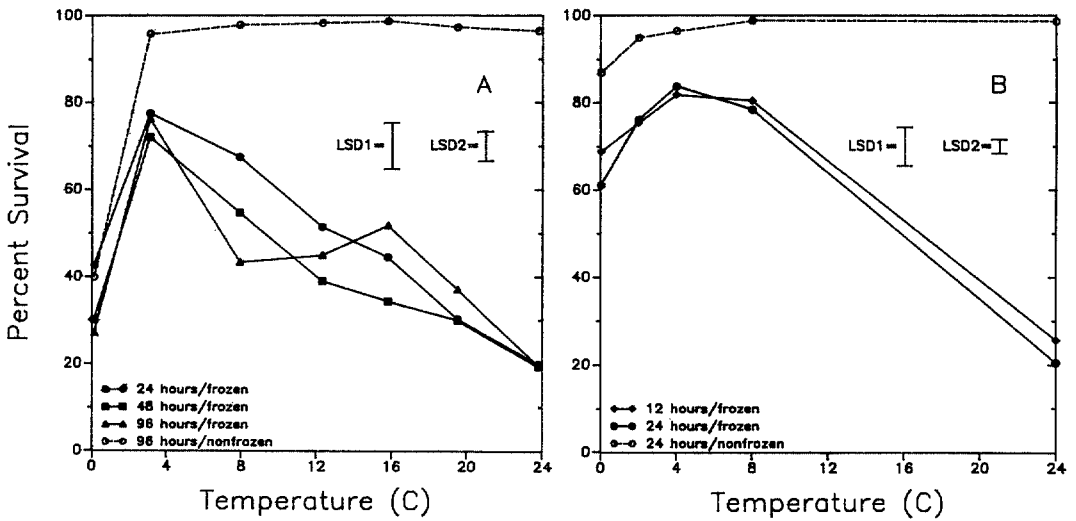


FIG. 1. Effect of prefreeze temperature and duration on percentage of juveniles that survived freezing at -4 C for 24 hours and survival percentage for nonfrozen controls. A) Experiment 1. B) Experiment 2. LSD1 and LSD2 are least significant differences for comparison of temperatures \times time combinations across temperatures and times within a temperature, respectively.

for each temperature in each block were treated as subsamples and their means were analyzed.

Each block of the soil experiment consisted of six subsamples for each temperature treatment. Paired *t*-tests conducted on subsample means were used to compare the two prefreeze temperature treatments for survival percentage from frozen samples, survival percentage from nonfrozen controls, and total number of nematodes recovered.

RESULTS

Effects of prefreeze temperature: Freezing tolerance varied inversely with prefreeze temperatures of 4–24 C (Fig. 1). Interaction between the temperature and duration of prefreeze exposure occurred in both experiments ($P = 0.001$ and $P = 0.04$ for experiments 1 and 2, respectively). Juvenile survival was lower ($P < 0.01$) for prefreeze exposure to 0 C than to 4 C. Survival of nonfrozen controls exposed to 0 C was less ($P < 0.05$) than that of controls exposed to other temperatures.

Time course of increased freezing tolerance: Freezing tolerance was directly related to the duration of prefreeze exposure to 4 C

(Fig. 2). Freezing tolerance at each sample time was higher ($P < 0.05$) than that of the preceding sample time, except for the 9-hour and 12-hour samples. Freezing tolerance was inversely related to the duration of prefreeze exposure to 24 C. Freez-

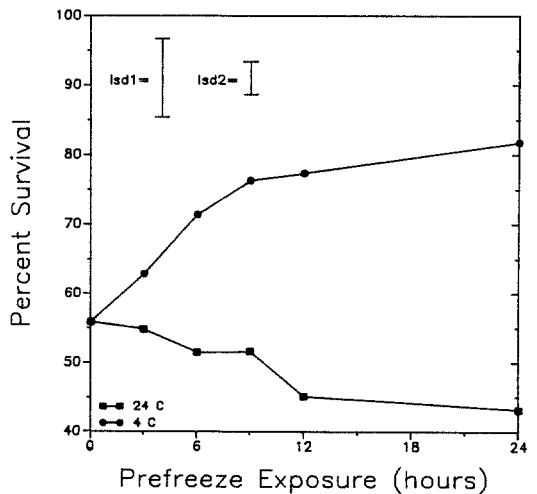


FIG. 2. Relationship between time at 4 or 24 C and percentage of juveniles that survived freezing at -4 C for 24 hours. LSD1 and LSD2 are least significant differences for comparison of temperature \times time combinations across temperatures and times within a temperature, respectively.

ing tolerance at the 12-hour and 24-hour sample times were lower ($P < 0.05$) than at the beginning of the experiment.

Increased freezing tolerance in soil: Survival after freezing was higher ($P < 0.01$) for juveniles that had a prefreeze exposure to 4 C ($25.7 \pm 7.3\%$) than for those exposed to 24 C ($13.5 \pm 7.7\%$). There was no difference between the two prefreeze exposures for survival of nonfrozen controls or for the total number of juveniles recovered.

DISCUSSION

Exposure to low temperatures increases the freezing tolerance of *M. hapla* juveniles. The increase in freezing tolerance, or cold hardening, is relatively rapid. Most research on invertebrate cold hardening has concentrated on biophysical and biochemical changes, with little emphasis on timing. When reported, the hardening periods typically range from weeks to months (1-4,15). In contrast, Lee et al. (8) reported significant cold hardening of nondiapausing pupae and adults of *Sarcophaga bulbata*, *S. crassipalpis*, *Xanthogaleruca luteola*, and *Oncopeltus fasciatus* after only 4 hours at 0 C. Both short-term (hourly or daily) and long-term (seasonal) changes in cold hardiness may affect the ecology of a species.

Exposure to low temperatures has been reported to lower the supercooling point of nematodes (2,9). Supercooling points have also been used as indicators of nematode cold tolerance (11,17). In a frozen environment, substantial mortality occurs at temperatures well above published supercooling points (Forge, unpubl.). Supercooling point is not a good indicator of hardiness for nematodes, and changes in supercooling point after exposure to low temperature may be a fortuitous result of the hardening process. We propose that an increased ability to survive frozen conditions is a better indicator of cold hardening than is a reduction in supercooling point.

The mortality of nonfrozen controls at 0 C was unexpected. Samples were examined at least four times daily between 6 a.m.

and 12 midnight, and ice was never observed. In addition, temperatures below 0.0 C were never recorded during the experiments. Because the mortality at 0 C cannot be explained by ice formation, we suggest that *M. hapla* juveniles may be sensitive to chilling at temperatures above the freezing point. Our current hypothesis is that previous exposure to temperatures such as 4 C increases chilling tolerance, just as it increases freezing tolerance.

In the first experiment, freezing tolerance of juveniles exposed to 24 C did not change between the 24-hour and 96-hour sample times. In the time course experiment, freezing tolerance of juveniles exposed to 24 C was significantly less at the 12-hour and 24-hour sample times than at previous sample times. Since the juveniles were reared and harvested at 24 C, the drop in freezing tolerance was not a delayed effect of temperature change. The higher freezing tolerance at early sample times may have been age related or caused by an event associated with sample preparation, such as collection in the Baermann funnel or rinsing.

In summary, we have demonstrated that *M. hapla* juveniles undergo cold hardening; when exposed to low temperatures, juveniles become more tolerant of a frozen environment. Most of the increase in freezing tolerance occurs within 12 hours at 4 C.

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